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13. ABSTRACT (Maximum 200 Words)

Retinoids are vitamin A derivatives, which cause growth inhibition, differentiation and/or apoptosis in various cell types, including some breast cancer cells. In general, estrogen receptor (ER)-positive cells are retinoic acid (RA) sensitive, whereas ER-negative cells are resistant. In this report, I show that ER-negative MDA-MB-231 cells are strongly growth inhibited by retinoids in combination with a PKC inhibitor. While neither RA nor GF109203X (GF has a significant growth inhibitory effect in these cells, RA+GF potently suppress proliferation and induce apoptosis Moreover, GF was found to enhance RA-induced transcriptional activation of an RARE reporter construct. Expression of phosphorylated as well as total PKC α and δ was decreased by GF and this was potentiated by RA. In addition, treatment with GF caused a sustained activation of ERK1/2 and p38-MAPK. Importantly, inhibition of ERK but not p38 or JNK suppressed apoptosis induced by RA+GF, indicating that activation of ERK is specifically required. In support of this novel finding, the ability of other PKC inhibitors to cause apoptosis in combination with RA correlates with ability to cause sustained activation of ERK. Moreover, it appears that inhibition/downregulation of PKCδ is specifically involved.

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Introduction

Retinoids are derivatives of vitamin A, which induce differentiation and growth inhibition in a variety of cell types, including breast cancer cells [1-3]. They act mainly by binding to nuclear retinoid receptors, RARs and RXRs, which act as ligand regulated transcription factors [4]. Several natural and synthetic retinoids can inhibit the development of mammary tumors and cause regression of established tumors in rats [5-7]. There is also clinical evidence that retinoids may be beneficial in breast cancer prevention [8, 9]. This encourages further studies of the potential use of retinoids in therapy of breast cancer. For this purpose, it is important to clarify how retinoids exert their effects on breast cancer cells and what determines sensitivity vs. resistance to these compounds.

Inhibition of growth by retinoic acid (RA) in human breast cancer cells *in vitro* generally correlates with expression of ERα. Most ERα-positive cell lines are growth inhibited in response to retinoids, whereas ERα-negative cells are resistant [10, 11]. We have previously shown that ERα modulates the transcriptional activity of the retinoic acid receptor (RAR) in a ligand independent manner, via its N-terminal AF-1 domain [12]. Furthermore, we have recently shown that stable overexpression of ERβ also restores sensitivity to RA in MDA-MB-231 cells.

There is also considerable evidence of crosstalk between retinoid signaling and signal transduction pathways activated by growth factors as well as stress stimuli. For example, there have been several reports of crosstalk, both positive and negative, between retinoids and PKC [13-16] as well as well as between retinoid signaling and the MAPK pathways [17-23]. In relation to this, I have found that several PKC inhibitors strongly potentiate the response of MDA-MB-231 to RA, and that this involves regulation of PKC as well as MAPK signaling (Oncogene, in press).

The approved Statement Of Work for this award proposes experiments described in two main tasks: **1) Define the signal transduction pathways whereby inhibitors of PKC restore sensitivity to retinoids in ER-negative cells**, and **2) Test the hypothesis that expression of ER alters the phosphorylation status of RAR and/or RXR, thereby rendering the cells sensitive to RA.** This annual report will focus mainly on the experiments proposed in Task 1, and I will describe results obtained in regard to regulation of PKC as well as MAPK signaling by RA and GF109203X in MDA-MB-231 breast cancer cells. I will also discuss our main findings that selective inhibition/downregulation of the PKCδ isoform seems to confer some retinoid sensitivity to MDA-MB-231 cells, but that *activation* of ERK1/2 is also required.

Body/Results

For details and figures see the appended article: Pettersson et al (2004), Oncogene, in press.

GF109203X enhances RAR mediated transcription from a transiently expressed promoter.

I had previously found that the broad range PKC inhibitor GF109203X (GF) could enhance the growth inhibitory/apoptotic response of MDA-MB-231 cells to RA (*Figure 1 and 2*). More recently, I also showed that GF induces apoptosis in combination with two synthetic retinoids, one RAR selective (TTNPB) and one more RXR selective (Bexarotene) (*Figure 3A*). Thus, to investigate if GF alters the transcriptional activity of the retinoic acid receptors, I begun by transiently transfecting MDA-MB-231 cells with reporter constructs containing an RARE and an RXR, respectively. These experiments showed that pretreatment with GF does indeed enhance

activation by RA of RAR mediated transcription. In contrast, activation of RXR mediated transcription by Bexarotene was unaffected by GF (*Figure 3B*).

RA and GF109203X downregulate PKC expression.

Since both RA and GF may regulate PKC expression and activity, I asked whether the synergistic growth inhibition and induction of apoptosis observed could be due to cooperative regulation of PKC by these agents. I assessed expression of phosphorylated PKC by Western blotting using an antibody specifically recognizing PKCα, βI, βII, ε, η, and δ when phosphorylated at a carboxy-terminal residue homologous to Ser660 of PKCβII (Cell Signaling Technology). In MDA-MB-231, I detected a single band, representing PKCα, which was downregulated by GF alone, and more strongly by RA+GF, after 48-72 hours of treatment. Moreover, I showed a decrease in total PKCα and δ protein levels. Northern blotting showed a slight downregulation of PKCα mRNA levels by both RA and GF, but no effect on PKCδ mRNA (*Figure 4 and data not shown*). In another ER-negative breast cancer cell line, MDA-MB-468, the main PKC isoform expressed is PKCδ. In agreement with results obtained in MDA-MB-231, this isoform was downregulated by GF, and more strongly by RA+GF (*data not shown*).

GF109203X causes sustained activation of MAPK pathways.

Several signal transduction pathways, including MAPK and AKT signaling, are regulated downstream of PKC. I used Western blotting with phospho-specific antibodies, which only react with the active forms of the respective proteins, to evaluate ERK1/2, p38 and AKT activity after treatment with RA and GF. Furthermore, I used an immune-complex kinase assay to evaluate JNK activity. This revealed a strong and sustained *activation* of both ERK and p38 by GF that was evident after 3 h (ERK) and 24 h (p38) respectively (*Figure 5A and 6*). In contrast, JNK was weakly activated by both RA and JNK (*Figure 5B*). AKT activity was found to be slightly decreased by RA alone, but GF did not potentiate this effect, leading us to conclude that regulation of AKT is not important for the synergistic growth inhibitory effect observed with RA+GF (*not shown*).

Inhibition of the MEK/ERK pathway blocks induction of apoptosis.

To further examine the role of MAPK activation in growth inhibition/apoptosis induced by RA+GF, MDA-MB-231 cells were cotreated with RA, GF and selective MAPK inhibitors. Remarkably, two different MEK inhibitors, which specifically block downstream activation of ERK, blocked induction of apoptosis by RA+GF. On the other hand, a p38 selective inhibitor and a JNK selective inhibitor did not inhibit apoptosis. (*Figure 7 and data not shown*)

The ability of other PKC inhibitors to inhibit cell proliferation and induce apoptosis in combination with RA correlates with ability to activate ERK.

In addition to GF, I tested three PKC inhibitors for their ability to induce apoptosis in combination with RA in MDA-MB-231. Two of those, Go6983 and Rottlerin, did induce significant apoptosis when combined with RA, while having a much weaker effect as single agents. On the other hand, UCN-01 alone was strongly growth inhibitory, and did not act in an additive or synergistic manner with RA at any concentrations tested. When I assessed the regulation of ERK by these three agents, I found that Go6983 and Rottlerin both activated ERK

while UCN-01 did not, consistent with ability/inability to cause apoptosis when combined with RA. (*Figure 8*)

Downregulation of PKCδ using siRNA confers partial responsiveness to RA.

GF109203X and Go6983 are broad-range PKC inhibitors that inhibit all PKC isoforms [24, 25]. UCN-01, on the other hand, inhibits mainly PKC$\alpha/\beta/\gamma$ [26] and Rottlerin is a PKCδ selective inhibitor [27]. Therefore, I decided to examine the effect of selectively downregulating PKCα or δ using siRNA. Efficient and selective downregulation of both isoforms was achieved, but only siRNA targeted to PKCδ lead to a significant increase in the percentage of apoptotic cells upon treatment with RA (*Figure 9*). However, RA+GF still caused a significantly stronger response than RA alone in these cells, indicating that GF produces an additional effect that also promotes apoptosis. Based on the evidence presented above, I suggest that this effect consists of activation of ERK. Of note, downregulation of either PKCα or δ did not alter ERK activity (*data not shown*).

Deviations from the approved Statement Of Work

- Task 1a: To assess PKC activity, I chose to do Western analysis using phospho-specific antibodies, since I found that the SignaTECT PKC system produced very variable results. Western analysis was performed using phospho-specific pan-PKC as well as PKCα and δ selective antibodies. Translocation of PKCα and δ from cytosol to membrane has not been assessed.
- Task 1c: Regulation of ERK, p38 and JNK has already been assessed for MDA-MB-231.
- Task 1d: Transient transfections assessing the effect of GF109203X on RAR transcriptional activity have already been performed.
- Task 1f: I have already tested the ability of UCN-01 to restore RA response in MDA-MB-231 and defined its effect on PKC and ERK activity.
- In addition to the experiments proposed in Task 1, and as an alternative to the dominant negative constructs described in Task 1b, I performed siRNA experiments to transiently downregulate PKCα and δ.

List of Key Research Accomplishments

- I have shown that several PKC inhibitors, including GF109203X, Go6983 and Rottlerin, enhance retinoid-induced apoptosis.
- I have shown that GF109203X enhances RAR mediated transcription form a transiently expressed promoter.
- I have shown that RA and GF109203X downregulate the levels of total and phosphorylated PKCα and δ and that this regulation occurs mainly at the protein level.
- I have shown that GF109203X, Go6983 and Rottlerin induced a sustained activation of MAPK signaling.
- I have identified activation of ERK1/2 MAPK signaling as crucial for the ability of PKC inhibitors to stimulate apoptosis in response to RA.
- I have identified PKCδ as the isoform whose downregulation is most likely to be involved in enhancing apoptosis in response to RA.

- I have begun to assess PKC and MAPK activity, as well as phosphorylation of RARα, in MDA-MB-231 stably expressing ERα and ERβ. This will start to address the possible role of these signaling pathways in the regulation of RAR activity by the estrogen receptors.

Reportable outcomes

A) Peer-reviewed articles:
F Pettersson, M-C Couture, Nessrine Hanna, and WH Miller, Jr. *Enhanced retinoid-induced apoptosis of MDA-MB-231 breast cancer cells by PKC inhibitors involves activation of ERK.* **Oncogene**, in press, 2004

B) Presentations at international conferences:
F Pettersson, M-C Couture, and W H Miller, Jr. *Synergistic induction of apoptosis in MDA-MB-231 breast cancer cells by retinoids and inhibitors of PKC requires activation of ERK 1/2.* I was selected for a short oral presentation at a minisymposium, AACR 94th annual meeting, Washington DC, July 2003. **Proc. Am. Ass. Cancer Res. (2d ed.) July 2003.**

F Pettersson, M-C Couture, N Hanna, and W H Miller, Jr. *Enhanced retinoid-induced apoptosis of MDA-MB-231 breast cancer cells by PKC inhibitors involves activation of ERK.* Poster presentation, 12th international conference on Second Messengers and Phosphoproteins, Montreal, August 2004. I was selected for a free registration award by the McGill Cancer Center.

Conclusions

This project has progressed in a satisfactory manner and no major problems were encountered during year 1. My work has produced several interesting pieces of data, and has resulted in one peer-reviewed publication to date.

References

1. Rubin, M, Fenig, E, Rosenauer, A, Menendez-Botet, C, Achkar, C, Bentel, J M, Yahalom, J, Mendelsohn, J and Miller, W H, Jr., *9-Cis retinoic acid inhibits growth of breast cancer cells and down-regulates estrogen receptor RNA and protein.* Cancer Research, 1994. **54**(24): p. 6549-6556.
2. Mangiarotti, R, Danova, M and Alberici, C, *All-trans retinoic acid (ATRA)-induced apoptosis is preceded by G1 arrest in human MCF-7 breast cancer cells.* Br J Cancer, 1998. **77**(2): p. 186-191.
3. Zhu, W Y, Jones, C S, Kiss, A, Matsukuma, K, Amin, S and De, L L, *Retinoic acid inhibition of cell cycle progression in MCF-7 human breast cancer cells.* Exp.Cell Res, 1997. **234**(2): p. 293-299.
4. Evans, R M, *The steroid and thyroid hormone receptor superfamily.* Science, 1988. **240**: p. 889-895.
5. Bischoff, E D, Gottardis, M M, Moon, T E, Heyman, R A and Lamph, W W, *Beyond tamoxifen: the retinoid X receptor-selective ligand LGD1069 (TARGRETIN) causes complete regression of mammary carcinoma.* Cancer Res, 1998. **58**(3): p. 479-484.
6. Gottardis, M M, Lamph, W W, Shalinsky, D R, Wellstein, A and Heyman, R A, *The efficacy of 9-cis retinoic acid in experimental models of cancer.* Breast Cancer Res Treat, 1996. **38**(1): p. 85-96.
7. Thompson, H J, Becci, P J, Moon, R C, Sporn, M B, Newton, D L, Brown, C C, Nurrenbach, A and Paust, J, *Inhibition of 1-methyl-1-nitrosourea-induced mammary carcinogenesis in the rat by the retinoid axerophthene.* Arzneimittelforschung, 1980. **30**(7): p. 1127-1129.
8. Hunter, D J, Manson, J E, Colditz, G A, Stampfer, M J, Rosner, B, Hennekens, C H, Speizer, F E and Willett, W C, *A prospective study of the intake of vitamins C, E, and A and the risk of breast cancer.* N Engl J Med, 1993. **329**(4): p. 234-240.
9. Veronesi U, D P G, Marubini E et al., *Randomized trial of fenretinide to prevent second breast malignancy in women with early breast cancer.* Journal of the National Cancer Institute, 1999. **91**(21): p. 1847-1856.

10. Sheikh, M S, Shao, Z M, Li, X S, Ordonez, J V, Conley, B A, Wu, S, *et al.*, *N-(4-hydroxyphenyl)retinamide (4-HPR)-mediated biological actions involve retinoid receptor-independent pathways in human breast carcinoma.* Carcinogenesis, 1995. **16**(10): p. 2477-2486.
11. Rosenauer, A, Nervi, C, Davison, K, Lamph, W W, Mader, S and Miller, W H, Jr., *Estrogen receptor expression activates the transcriptional and growth-inhibitory response to retinoids without enhanced retinoic acid receptor alpha expression.* Cancer Research, 1998. **58**(22): p. 5110-5116.
12. Rousseau, C, Pettersson, F, Couture, M C, Paquin, A, Galipeau, J, Mader, S and Miller, W H, *The N-terminal of the estrogen receptor (ERalpha) mediates transcriptional cross-talk with the retinoic acid receptor in human breast cancer cells.* J Steroid Biochem Mol Biol, 2003. **86**(1): p. 1-14.
13. Delmotte, M-H, Tahayato, A, Formstecher, P and Lefebvre, P, *Serine 157, a retinoic acid receptor α residuephosphorylated by protein kinase C in vitro, is involved in RXR-RARα heterodimerisatoion and transcriptional activity.* J Biol Chem, 1999. **274**(53): p. 38225-38231.
14. Cho, Y, Tighe, A P and Talmage, D A, *Retinoic acid induced growth arrest of human breast carcinoma cells requires protein kinase C alpha expression and activity.* J Cell Physiol, 1997. **172**(3): p. 306-13.
15. Cho, Y and Talmage, D A, *Protein kinase Calpha expression confers retinoic acid sensitivity on MDA-MB-231 human breast cancer cells.* Exp Cell Res, 2001. **269**(1): p. 97-108.
16. Kambhampati, S, Li, Y, Verma, A, Sassano, A, Majchrzak, B, Deb, D K, *et al.*, *Activation of protein kinase C-delta (PKC-delta) by All-trans-retinoic acid.* J Biol Chem, 2003. **278**(35): p. 32544-51.
17. Li, D, Zimmerman, T L, Thevananther, S, Lee, H Y, Kurie, J M and Karpen, S J, *Interleukin-1beta -mediated Suppression of RXR:RAR Transactivation of the Ntcp Promoter Is JNK-dependent.* J Biol Chem, 2002. **277**(35): p. 31416-22.
18. Lee, H-J, Suh, Y-A, Robinson, M J, Clifford, J L, Hong, W K, Woodgett, J R, Cobbs, M H, Mangelsdorf, D J and Kurie, J M, *Stress pathway activation induces phosphorylation of retinoid X receptor.* J Biol Chem, 2000. **275**(41): p. 32193-32199.
19. Solomon, C, White, J H and Kremer, R, *Mitogen-activated protein kinase inhibits 1,25-dihydroxyvitamin D3-dependent signal transduction by phosphorylating human retinoid X receptor α.* J Clin Invest, 1999. **103**: p. 1729-1735.
20. Matsushima-Nishiwaki, R, Okuno, M, Adachi, S, Sano, T, Akita, K, Moriwaki, H, Friedman, S L and Kojima, S, *Phosphorylation of retinoid X receptor alpha at serine 260 impairs its metabolism and function in human hepatocellular carcinoma.* Cancer Res, 2001. **61**(20): p. 7675-82.
21. Miranda, M B, McGuire, T F and Johnson, D E, *Importance of MEK-1/-2 signaling in monocytic and granulocytic differentiation of myeloid cell lines.* Leukemia, 2002. **16**(4): p. 683-92.
22. Yen, A, Roberson, M S, Varvayanis, S and Lee, A T, *Retinoic acid induced mitogen-activated protein (MAP)/extracellular signal-regulated kinase (ERK) kinase-dependent MAP kinase activation needed to elicit HL-60 cell differentiation and growth arrest.* Cancer Res, 1998. **58**(14): p. 3163-72.
23. Bost, F, Caron, L, Marchetti, I, Dani, C, Le Marchand-Brustel, Y and Binetruy, B, *Retinoic acid activation of the ERK pathway is required for embryonic stem cell commitment into the adipocyte lineage.* Biochem J, 2002. **361**(Pt 3): p. 621-7.
24. Toullec, D, Pianetti, P, Coste, H, Bellevergue, P, Grand-Perret, T, Ajakane, M, *et al.*, *The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C.* J Biol Chem, 1991. **266**(24): p. 15771-15781.
25. Gschwendt, M, Dieterich, S, Rennecke, J, Kittstein, W, Mueller, H J and Johannes, F J, *Inhibition of protein kinase C mu by various inhibitors. Differentiation from protein kinase c isoenzymes.* FEBS Lett, 1996. **392**(2): p. 77-80.
26. Hofmann, J, *Modulation of protein kinase C in antitumor treatment.* Rev Physiol Biochem Pharmacol, 2001. **142**: p. 1-96.
27. Gschwendt, M, Muller, H J, Kielbassa, K, Zang, R, Kittstein, W, Rincke, G and Marks, F, *Rottlerin, a novel protein kinase inhibitor.* Biochem Biophys Res Commun, 1994. **199**(1): p. 93-8.

Appendices

Article: **F Pettersson**, M-C Couture, Nessrine Hanna, and WH Miller, Jr. *Enhanced retinoid-induced apoptosis of MDA-MB-231 breast cancer cells by PKC inhibitors involves activation of ERK.* **Oncogene**, in press, 2004 (uncorrected proofs).

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ORIGINAL PAPER

Enhanced retinoid-induced apoptosis of MDA-MB-231 breast cancer cells by PKC inhibitors involves activation of ERK

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Retinoids are vitamin A derivatives, which cause growth inhibition, differentiation and/or apoptosis in various cell types, including some breast cancer cells. In general, estrogen receptor (ER)-positive cells are retinoic acid (RA) sensitive, whereas ER-negative cells are resistant. In this report, we show that ER-negative MDA-MB-231 cells are strongly growth inhibited by retinoids in combination with a PKC inhibitor. While neither RA nor GF109203X (GF) has a significant growth inhibitory effect in these cells, RA + GF potently suppress proliferation. We found that RA + GF induce apoptosis, as shown by an increase in fragmented DNA, Annexin-V-positive cells and caspase-3 activation. Apoptosis was also induced by GF in combination with two synthetic retinoids. Expression of phosphorylated as well as total PKC was decreased by GF and this was potentiated by RA. In addition, treatment with GF caused a strong and sustained activation of ERK1/2 and p38-MAPK, as well as a weaker activation of JNK. Importantly, inhibition of ERK but not p38 or JNK suppressed apoptosis induced by RA + GF, indicating that activation of ERK is specifically required.In support of this novel finding, the ability of other PKC inhibitors to cause apoptosis in combination with RA correlates with ability to cause sustained activation of ERK.
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Keywords: ER-negative breast cancer; retinoids; protein kinase C inhibitors; MAPK; apoptosis

Introduction

Breast cancer is an increasingly common malignancy with few curative options beyond early detection. In particular, estrogen receptor (ER)-negative breast cancers are unresponsive to antiestrogen therapy and generally more aggressive than ER-positive tumors (Rochefort *et al.*, 2003). Development of novel, more active chemotherapeutic agents or combination therapies will therefore be an important step in the battle against this disease.

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Retinoids are derivatives of vitamin A, which have shown antitumor activity in a variety of *in vitro* and *in vivo* models (De Luca *et al.*, 1995; Lippman *et al.*, 1995; Lotan, 1996; Miller, 1998). All-*trans*-retinoic acid (RA), a natural and hormonally active vitamin A metabolite, is used in clinical treatment of acute promyelocytic leukemia (APL), and synthetic retinoids are currently in clinical trials for the treatment of other malignancies, including breast cancer.

The molecular mechanisms by which retinoids exert their antiproliferative effects are not fully elucidated, but involve binding to the nuclear retinoic acid receptors, RARs and RXRs. These receptors form RAR/RXR heterodimers or RXR homodimers, which function as ligand-induced transcription factors that switch a variety of genes on and off (Evans, 1988; Mangelsdorf *et al.*, 1995). Growth inhibition of breast carcinoma cells by RA has been shown to be mediated mainly by activation of the RARα isoform (van der Burg *et al.*, 1993, 1995; Fitzgerald *et al.*, 1997), and to involve downregulation of the estrogen receptor (ERα) and ER target genes (Rubin *et al.*, 1994), as well as degradation of cyclin D1 (Zhu *et al.*, 1997). We have also recently reported the importance of a decrease in IRS-1 levels and downstream signaling through the PI3-K/AKT pathway following treatment with RA in the ER-positive cell line MCF-7 (del Rincon *et al.*, 2003).

ER-negative breast cancer cells are generally resistant to retinoids, and this has been linked to downregulation of RARα levels (Sheikh *et al.*, 1993, 1994). However, we have previously reported that sensitivity to RA can be restored in the ER-negative MDA-MB-231 cell line by stable reintroduction of ERα (Rosenauer *et al.*, 1998) or ERβ (manuscript submitted), without an increase in RARα levels. Thus, the mechanism of RA-resistance in this cell line is not clear. Given the increasing evidence that shows crosstalk between the activity of nuclear receptors and signal transduction pathways, such as the protein kinase C (PKC), the MAPK and the PI3-K/AKT pathways, we investigated whether altered activity of these pathways may play a role.

PKC represents a family of serine/threonine kinases, which control signal transduction pathways involved in regulation of cell growth, survival and differentiation. The PKC family comprises at least 12 isoforms, the conventional cPKCs (α, βI, βII and γ), the novel nPKCs (δ, ε, η, μ and θ) and the atypical aPKCs (λ, ι and ζ).

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Characteristic for all PKC isoforms is their dependence on lipids (phosphatidylserine, PS) for activity. The cPKCs also require diacylglycerol (DAG) and Ca^{2+} for their activation, whereas the nPKCs are Ca^{2+} independent and the aPKCs are independent of both Ca^{2+} and DAG (Mellor and Parker, 1998; Martelli *et al.*, 1999). Moreover, activation of the PKC proteins is regulated by three distinct phosphorylation events, in the activation domain, at an autophosphorylation site and (except for the aPKCs) at a hydrophobic C-terminal site (Keranen *et al.*, 1995; Parekh *et al.*, 2000).

There have been several reports of crosstalk, both positive and negative, between retinoids and PKC. Notably, both PKCα and γ can phosphorylate RARα on Ser157, in the extended DNA-binding domain, and this reduces its ability to form an active heterodimer with RXR (Delmotte *et al.*, 1999). However, PKCα can positively regulate RA-signaling and has been demonstrated to be required for growth inhibition by RA in some cells (Cho *et al.*, 1997; Cho and Talmage, 2001). PKCα expression is also increased by RA in many RA-sensitive cells (Boskovic *et al.*, 2002), although we have studied several breast cancer cells lines and found no correlation between expression or induction of PKCα and RA-sensitivity (unpublished observation). A positive interaction between RARα and PKCδ was also recently reported. The authors showed that RA can activate PKCδ in MCF-7 cells and that overexpression of PKCδ increases transcriptional activity from an RARE in these cells (Kambhampati *et al.*, 2003). On the other hand, RA has been reported to act as a specific ligand for PKCα *in vitro*, competing with the lipid activator PS, thereby acting as an *inhibitor* of PKC activity (Radominska-Pandya *et al.*, 2000). Moreover, it has been shown that the pan-PKC inhibitor staurosporine can potentiate induction of differentiation by RA (Yung and Hui, 1995).

There is also increasing evidence for crosstalk between retinoid signaling and the MAPK pathways (ERK, p38MAPK and JNK). For example, phosphorylation of RXRα by ERK and JNK can inhibit signaling by RA as well as RXR selective retinoids (Solomon *et al.*, 1999; Lee *et al.*, 2000a; Matsushima-Nishiwaki *et al.*, 2001; Li *et al.*, 2002). On the other hand, RA is known to induce the activation of ERK in certain cell types, and this has been shown to be required for some of its actions, including induction of differentiation and growth arrest (Yen *et al.*, 1998; Bost *et al.*, 2002; Miranda *et al.*, 2002).

We report here that three inhibitors of PKC, the pan-selective GF109203X (Toullec *et al.*, 1991; Gschwendt *et al.*, 1996) and Go6983 (Gschwendt *et al.*, 1996), and the PKCδ selective Rottlerin (Gschwendt *et al.*, 1994), have the ability to interact with retinoids in retinoid-resistant MDA-MB-231 cells and cause growth inhibition, partially by inducing apoptosis, in an additive or synergistic manner. We further present evidence that this involves a strong and sustained activation of ERK1/2.

Results

GF109203X and RA interact to inhibit proliferation and induce apoptosis in a synergistic manner

MDA-MB-231 cells were treated with RA, GF or RA + GF at the indicated concentrations for up to 8 days and total cell number was assessed as described in Materials and methods. Neither RA nor GF had a significant growth inhibitory effect as single agents at any time point. However, the combination caused a marked reduction in cell growth that was apparent by day 3. A maximal inhibition of 80% compared to untreated cells was achieved by day 8 (Figure 1). Propidium iodide staining and cell cycle analysis was then performed on cells treated for 24, 48 and 72 h. Treatment with RA + GF for 24 h showed no significant effect, whereas a 48-h treatment led to partial growth arrest in the G1 phase of the cell cycle (60 ± 1.5 *vs* 55 ± 1.4% for control-treated cells), accompanied by a decrease in S phase (32 ± 1.3 *vs* 37 ± 1.2%, $P < 0.05$). After 72 h, however, a significant increase in cells with sub-G1 DNA content was observed, indicating that the cells were undergoing apoptosis. This was observed for cells treated with GF in combination with 1 μM RA, but was more pronounced for cells treated with GF plus 10 μM RA (Figure 2a). Of note, RA alone did not induce apoptosis at any concentration used. To confirm that the cells were indeed undergoing apoptosis, several methods were used. Activation of effector caspases by RA + GF was shown using a caspase-3 activity assay (Figure 2b), as well as by the detection of cleavage of the caspase-3/-7 substrate PARP (Figure 2c). Moreover, membrane changes associated with apoptosis were detected by Annexin V staining (Figure 2d).

To determine if the combination of RA and GF also induced differentiation of the tumor cells, we examined production of lipid droplets by Oil Red O staining (Le *et al.*, 2000; Adan *et al.*, 2003) in cells treated with RA, GF or RA + GF for up to 5 days. However, no evidence of differentiation was noted (not shown).

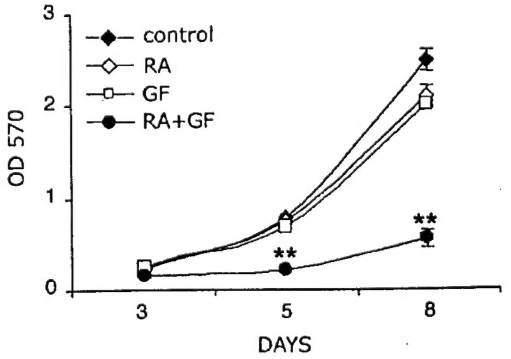

Figure 1 GF109203X and RA synergistically inhibit MDA-MB-231 cell proliferation. The cells were treated with 10 μM RA and 1 μM GF109203X and cell number was assessed using SRB staining as described in Materials and methods. Results from a representative experiment is shown as means ± s.d. **Indicates a significant difference compared to *all* treatments

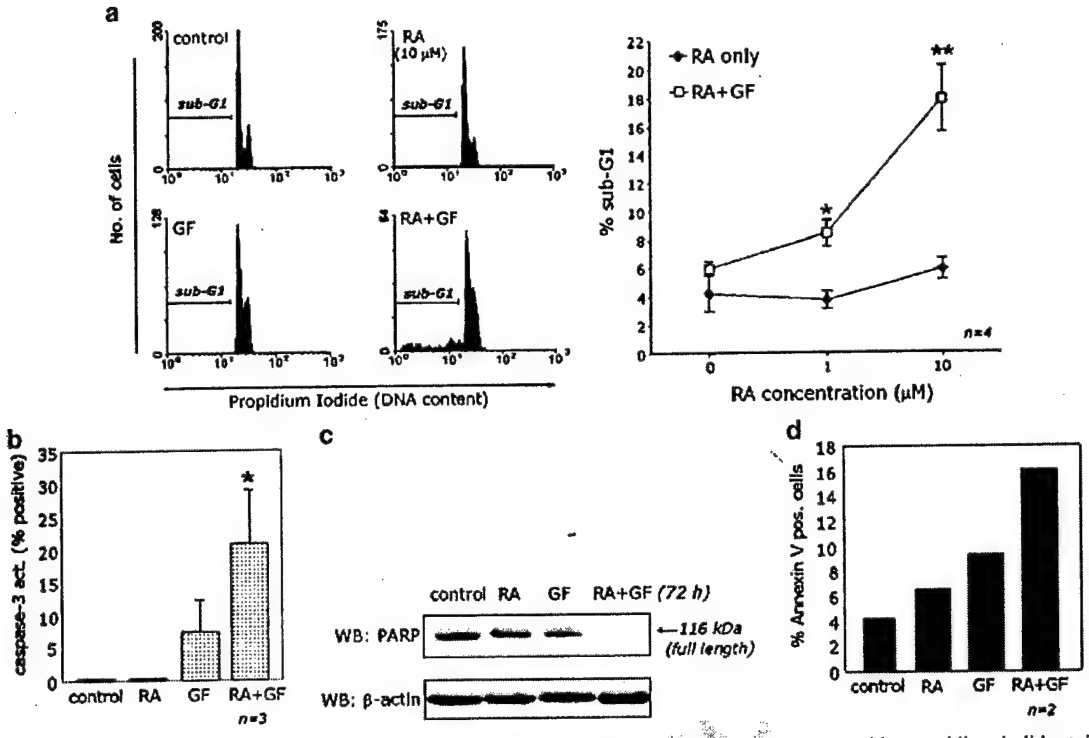

Figure 2 GF109203X and RA induce apoptosis in MDA-MB-231. (a) DNA fragmentation was assessed by propidium iodide staining and flow cytometry analysis, where cells with sub-G1 DNA content represent apoptotic cells. (b) Activation of caspase-3 was measured as binding of the fluorescent inhibitor Red-DEVD-FMK and analysis by flow cytometry. (c) Also shown is a Western blot indicating PARP cleavage in the presence of RA + GF. (d) Finally, apoptosis was shown by Annexin V staining, which measures the externalization of phosphatidylserine. In all experiments, the cells were treated for 72 h. $*P<0.05$ and $**P<0.01$ indicate significant differences compared to control

GF109203X induces apoptosis in combination with synthetic retinoids

To test whether the effect of GF is specific for RA, cells were treated with GF in combination with two synthetic retinoids. The cells were treated with an RAR-selective retinoid, TTNPB (LG100272), and the more RXR-selective retinoid, Bexarotene (Targretin, LGD1069), in the absence or presence of GF. Apoptosis was assessed by PI staining after 72 h, and we show that treatment with TTNPB + GF as well as Bexarotene + GF leads to induction of apoptosis, whereas the retinoids alone have no effect (Figure 3a). The level of apoptosis induced was comparable to that induced by RA + GF, indicating that activation of RAR/RXR heterodimers (by RA and TTNPB) or RXR homodimers (Bexarotene) may be involved.

GF enhances RAR-mediated transcription

The fact that RAR selective as well as RXR selective retinoids were shown to cause apoptosis only when combined with GF led us to examine whether GF could alter transcriptional activation from transiently expressed retinoid response elements. Indeed, pretreatment with GF was found to significantly enhance activation by RA of the βRARE-tk-CAT reporter (Rousseau *et al*., 2003), (Figure 3b). We also assessed transcriptional activation of CRBPII-tk-CAT, containing the RXRE from the CRBPII promoter (Mangelsdorf *et al*., 1991), by Bexarotene +/− GF. In contrast to its positive effect on βRARE-driven transcription, GF did not affect activation of CRBPII-tk-CAT (Figure 3b). GF also had no effect on the expression levels of RARα, RARβ or RXRα (data not shown).

GF109203X and RA downregulate PKC expression

Since RA as well as GF can regulate PKC expression and activity, we asked whether growth inhibition and apoptosis may be stimulated through cooperative regulation of PKC by these agents. Expression of phosphorylated PKC was assessed by Western blotting using a phospho-specific PKC antibody that detects PKCα, *βI*, *βII*, ε, η and δ only when phosphorylated at a carboxy-terminal residue homologous to Ser660 of PKC*βII*. A single band was detected at an apparent molecular weight of 82 kDa, which represents PKCα, the main isoform expressed in MDA-MB-231 (Platet *et al*., 1998; Gauthier *et al*., 2003). Moreover, in cells treated with GF, a decrease in phosphorylated PKC could be seen. RA alone had no significant effect, but the combination of RA + GF caused a stronger decrease than GF alone (Figure 4, panel 1). Expression levels of total PKCα and δ were also examined and it was found that both isoforms were downregulated by GF, and

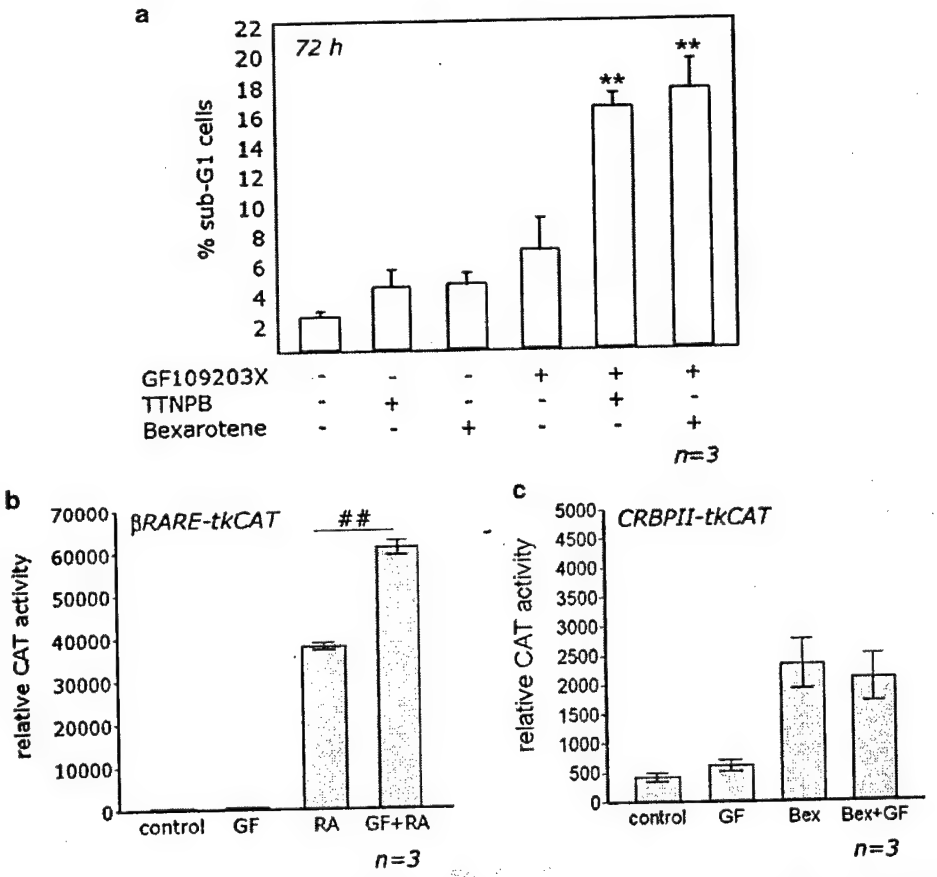

Figure 3 GF109203X interacts both with the RAR-selective retinoid TTNPB and the RXR-selective Bexarotene to induce apoptosis in MDA-MB-231 and RAR-mediated transcription is enhanced by GF. (**a**) The cells were treated with 5 μM of the retinoids and 1 μM GF109203X for 72 h and DNA fragmentation was assessed by propidium iodide staining. **Indicates a significant difference compared to control, GF alone and retinoid alone ($P<0.01$). (**b**) Transcriptional activity was assessed as described in Materials and methods. Results of one representative experiment are shown as means $\pm$ s.e.m. $^{\#\#}P<0.01$ indicates a significant difference between RA and RA + GF

more strongly by RA + GF (Figure 4, panels 2 and 3). RA alone also caused a small decrease in PKCα levels, but had no effect on PKCδ. Moreover, we assessed regulation of PKC mRNA levels and found that while PKCα was slightly downregulated by both RA and GF, PKCδ was unaffected (data not shown).

GF109203X causes sustained activation of MAPK pathways

We further examined regulation by RA and GF of signal transduction pathways that lie downstream of PKC. By Western analysis using phospho-specific antibodies, which only react with the activated forms of the proteins, we showed that both ERK1/2 and p38 MAPK activity was significantly *increased* in MDA-MB-231 cells treated with GF or RA + GF for 72 h (Figure 5a). Using a kinase assay, as described in Materials and methods, it was also shown that RA and GF caused a much weaker activation of JNK (Figure 5b). Total levels of ERK, p38 and JNK remained unchanged.

To determine at what time the observed changes in MAPK activity occurred, time-course experiments were performed. We found that ERK was already activated after a 3-h treatment with GF or RA + GF and that this activation remained high throughout the experiment (Figure 6, panel 1). In contrast, significant activation of p38 MAPK was observed only after 24 h, and remained high at 48 and 72 h (Figure 6, panel 3). Levels of phosphorylated PKC were also examined, and were found to decrease after 48–72 h (Figure 6, panel 4).

Inhibition of the MEK/ERK pathway blocks induction of apoptosis

To further investigate the role of MAPK activation in apoptosis induced by RA + GF, MDA-MB-231 cells were cotreated with RA, GF and selective MAPK inhibitors for up to 72 h. Remarkably, cotreatment with two different MEK inhibitors, PD98059 and U0126, which specifically block downstream activation of ERK1/2, almost completely blocked the induction of apoptosis by RA + GF. This was shown by PI staining

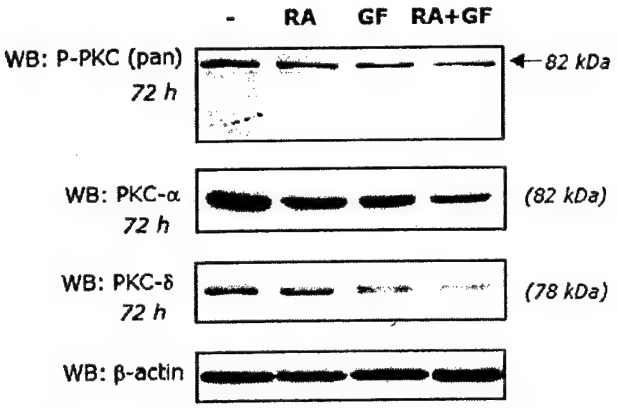

Figure 4 GF109203X and RA downregulate PKCα and δ. Expression of phosphorylated PKC was assessed by Western blotting using a phospho-specific PKC antibody. Total levels of PKCα and δ were detected using isoform-specific antibodies and a β-actin antibody was used to confirm equal loading. Representative blots using whole cell extracts from MDA-MB-231 treated with 10 μM RA and 1 μM GF109203X for 72 h are shown

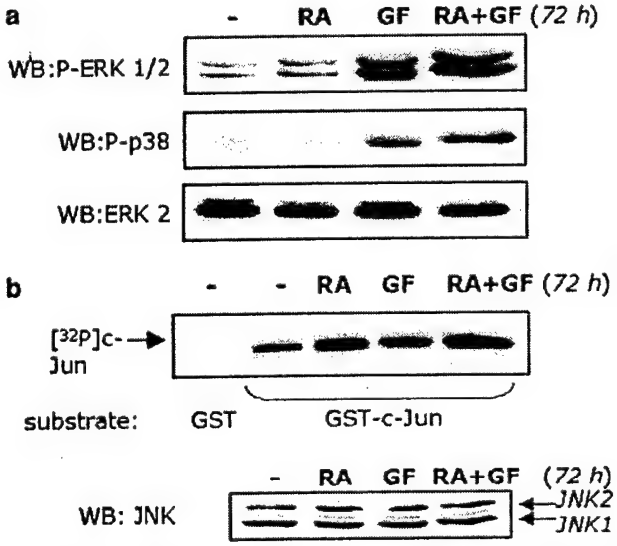

Figure 5 GF109203X induces a sustained activation of MAPK pathways in MDA-MB-231. (**a**) Active ERK and p38 were detected in whole cell lysates from cells treated as in Figure 4, by Western blotting using phospho-specific antibodies. Expression of total ERK2 did not change and was used to confirm equal loading. (**b**) JNK activity was assessed by a kinase assay as described in Materials and methods, using GST c-Jun as the substrate. Expression of total JNK was assessed by Western blotting

(Figure 7a) as well as Annexin V (Figure 7b). Moreover, addition of PD98059 increased the total cell number (live *plus* dead cells), compared to RA + GF (Figure 7c), indicating that the MEK inhibitor did not simply inhibit apoptosis by growth arresting the cells. This was further confirmed by cell cycle analysis, which showed no effect of PD98059 alone on cell cycle distribution (data not shown). Western blot analysis confirmed that PD98059 and U0126 inhibited activation of ERK under the conditions used (Figure 7d).

The cells were also cotreated with RA, GF and the p38-MAPK inhibitor SB203580, but contrary to the effect of the MEK inhibitors, SB203580 did not block induction of apoptosis. A concentration of 1 μM SB203580 had no effect on apoptosis (not shown) and a concentration of 5 μM increased, rather than decreased, the level of apoptosis observed (Figure 7e). An inhibitor of JNK, SP600125 (5 μM), had no significant effect on induction of apoptosis by RA + GF (data not shown). In addition, we assessed whether the MEK inhibitors had an effect on levels of phosphorylated PKC, and found that both PD98059 and U0126 rescued the downregulation caused by RA + GF (Figure 7f).

Ability of other PKC inhibitors to inhibit cell proliferation and induce apoptosis in combination with RA correlates with ability to activate ERK

Having established that GF can interact with RA to induce apoptosis in MDA-MB-231 cells, and that activation of ERK plays an important role, we wished to investigate if this phenomenon is unique to GF or applies to other inhibitors of PKC as well. Three different inhibitors, Go6983 (Go), UCN-01 and Rottlerin were examined for their ability to interact with RA and cause growth inhibition and apoptosis in MDA-MB-231 cells. Go6983 is a broad-range PKC inhibitor that is similar to GF, whereas UCN-01 inhibits mainly classical PKCs (Hofmann, 2001), and Rottlerin is selective for PKCδ (Gschwendt *et al*., 1994). The cells were treated for up to 8 days with each of the inhibitors in the absence or presence of RA and cell proliferation and apoptosis were assessed. Like GF, Go had a weak growth inhibitory effect by itself, but strongly suppressed growth and induced apoptosis in combination with RA (Figure 8a). UCN-01 had more potent growth inhibitory activity as a single agent than GF or Go, but at no concentration tested did UCN-01 act in an additive or synergistic manner with RA to cause growth inhibition or apoptosis (Figure 8b). Rottlerin was also a fairly potent growth inhibitor and induced some apoptosis by itself. However, the combination of Rottlerin and RA caused a significantly enhanced inhibition of cell growth and a higher level of apoptosis (Figure 8c).

Whole cell extracts were also prepared and Western analysis was performed to assess activation of ERK1/2 by Go, UCN-01 and Rottlerin. Consistent with the ability to synergize with RA to induce apoptosis, both Go6983 and Rottlerin caused a sustained activation of ERK1/2 that was apparent within 3–6 h, while UCN-01 had no effect on phospho-ERK1/2 levels (Figure 8d).

Downregulation of PKCδ using siRNA confers partial responsiveness to RA

Since the PKCδ selective inhibitor Rottlerin was found to significantly enhance inhibition of cell growth and apoptosis in response to RA, we tested if selective

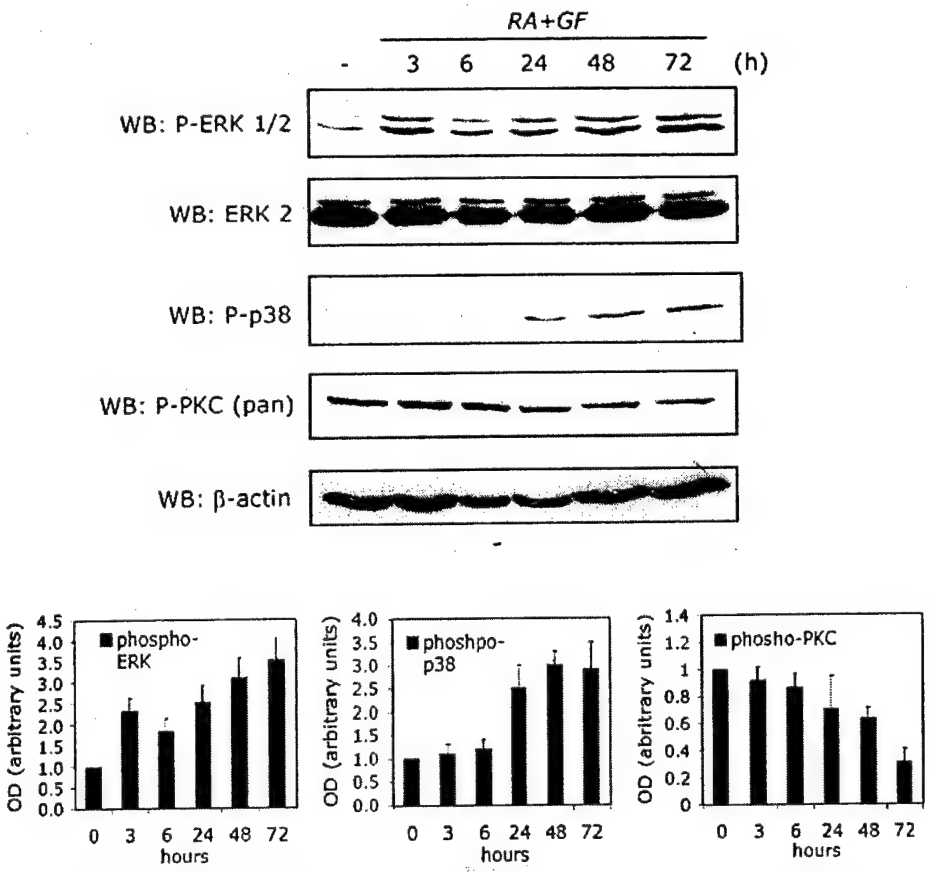

Figure 6 ERK activation occurs early and is sustained for several days, whereas p38 activation and PKC downregulation occurs later. The cells were treated with RA + GF for the indicated times and phosphorylated ERK, p38 and PKC were assessed by Western blotting as in Figures 4 and 5. Total ERK2 and β-actin were used as loading controls. All time-course experiments were performed three times and representative blots are shown. Densitometry was performed on three separate sets of blots to quantitate the signals obtained and histograms show optical density normalized against ERK2 (phospho-ERK) or β-actin (phospho-p38 and phospho-PKC)

downregulation of PKCα or δ using small interfering RNA (siRNA) could affect sensitivity to RA. It was observed that while downregulation of either PKCα or δ was somewhat toxic to the cells, only siRNA targeted to PKCδ conferred some responsiveness to RA. As shown in Figure 9, treatment with RA for 72 h following transfection with PKCδ siRNA led to a significant increase in the percentage of apoptotic cells. However, although PKCδ expression was almost completely abolished by siRNA, as determined by Western blotting (Figure 9a), RA + GF still caused a significantly stronger apoptotic response than RA alone. This indicates that treatment with GF produces an additional effect that also promotes apoptosis. Of note, downregulation of either PKCα or δ using siRNA did not alter phospho-ERK1/2 levels (data not shown).

Discussion

Retinoids have antiproliferative activity against breast cancer cells *in vitro* and *in vivo*. However, cells that lack expression of ER and cells with low expression of RARα are usually retinoid resistant. Moreover, clinical trials using retinoids in breast cancer patients have so far shown limited activity.

We report here that MDA-MB-231, an ER-negative and retinoid resistant cell line, can be growth inhibited by the combination of retinoids with pharmacological inhibitors of PKC. This potentiation of growth inhibition involves induction of apoptosis, and appears to be partially mediated through enhanced activation of RAR/RXR-mediated transcription. GF109203X was shown to enhance activation by RA of an RARE-driven reporter construct. In contrast, activation of an RXRE by the RXR selective Bexarotene was unaffected by GF, despite the fact that Bexarotene + GF induced apoptosis to the same extent as RA + GF. At the concentration used, it is known that Bexarotene also binds and activates RAR/RXR heterodimers, although with lower affinity than for RXR/RXR homodimers (Kizaki *et al*., 1996). Thus, the effect we observed may well be mediated via RAR-regulated transcription. In support of this, a ligand with higher selectivity for RXR, LG101305, failed to induce apoptosis when combined with GF (data not shown). However, effects that are independent of RAR- mediated transcription may also

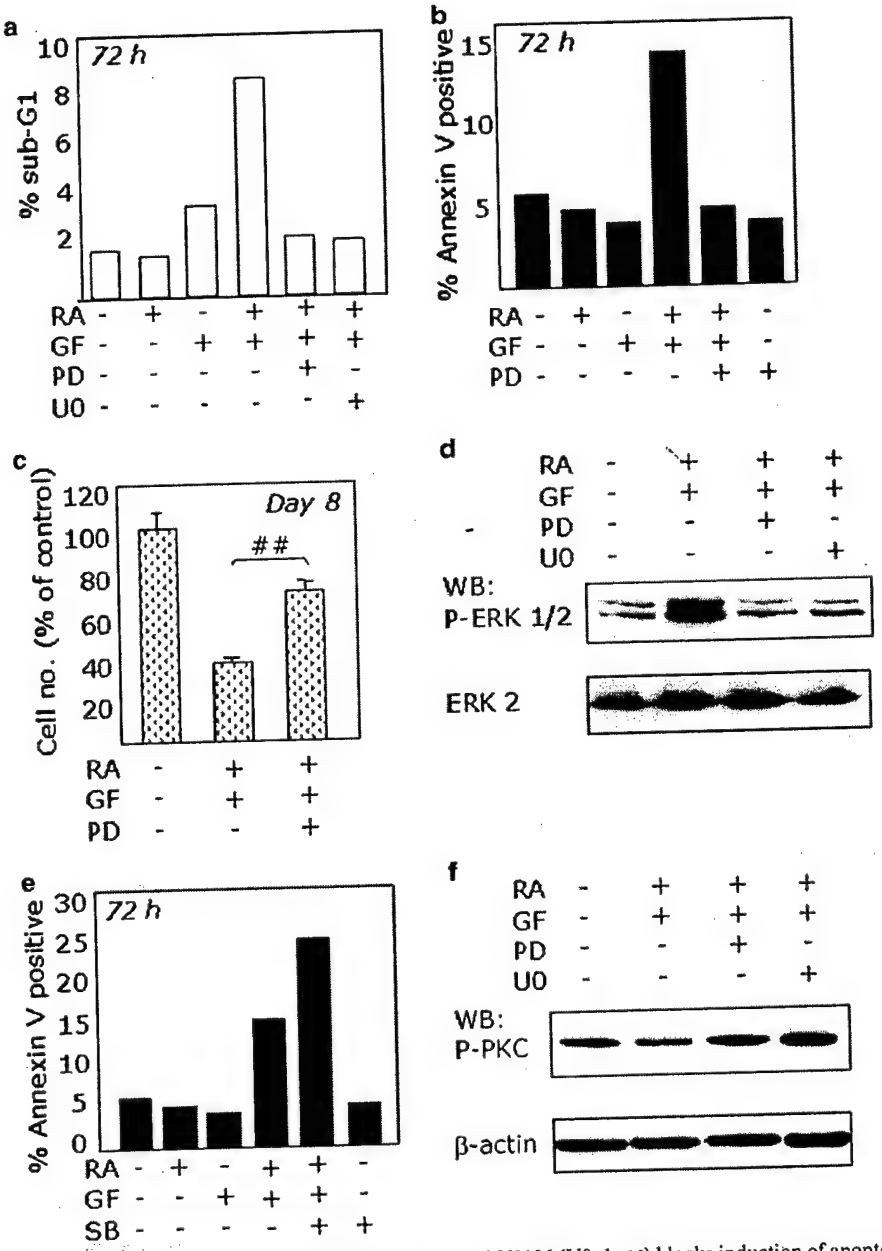

Figure 7 Cotreatment with the MEK inhibitors PD98059 (PD, 25 μM) and U0126 (U0, 1 μM) blocks induction of apoptosis by GF109203X + RA. (**a**) DNA content was assessed by propidium iodide staining on day 3, showing a near complete suppression of apoptosis by PD and U0. (**b**) Annexin-V staining confirmed that PD blocks induction of apoptosis by RA + GF, but has no effect on the basal level of apoptosis. (**c**) Assessment of total cell number (live *plus* dead cells) on day 8 of treatment showed that addition of PD also brings about an increase in total cell number. (**d**) Western analysis showed that PD and U0 block phosphorylation of ERK1/2 under the conditions used. (**e**) Cotreatment with the p38 inhibitor SB203580 (SB, 5 μM) does not block induction of apoptosis. (**f**) In addition to blocking apoptosis, PD and U0 prevent downregulation of phosphorylated PKC, as shown by Western blotting. Results from representative experiments are shown as mean values of two (a, b and e) or four (c) replicates. ##Indicates a significant difference between RA + GF and RA + GF + PD ($P<0.01$)

be involved, especially in cells treated with higher retinoid concentrations. Such effects have been reported to be responsible for the induction of apoptosis by synthetic retinoids such as Fenretinide (4-HPR) (Kitareewan *et al*., 1999; Wu *et al*., 2001) and CD437 (Sun *et al*., 1999b; Zhao *et al*., 2001), as well as by RA (Pepper *et al*., 2002). These include formation of DNA adducts, activation of stress kinase pathways, induction of reactive oxygen species and direct effects on mitochondria (Delia *et al*., 1995; Marchetti *et al*., 1999; Rigobello *et al*., 1999; Sun *et al*., 1999a; Hail *et al*., 2001; Ortiz *et al*., 2001; Zhao *et al*., 2001; Appierto *et al*., 2004).

We demonstrated that several PKC inhibitors could interact with retinoids to induce apoptosis in MDA-MB-231. There is some contradictory information in the

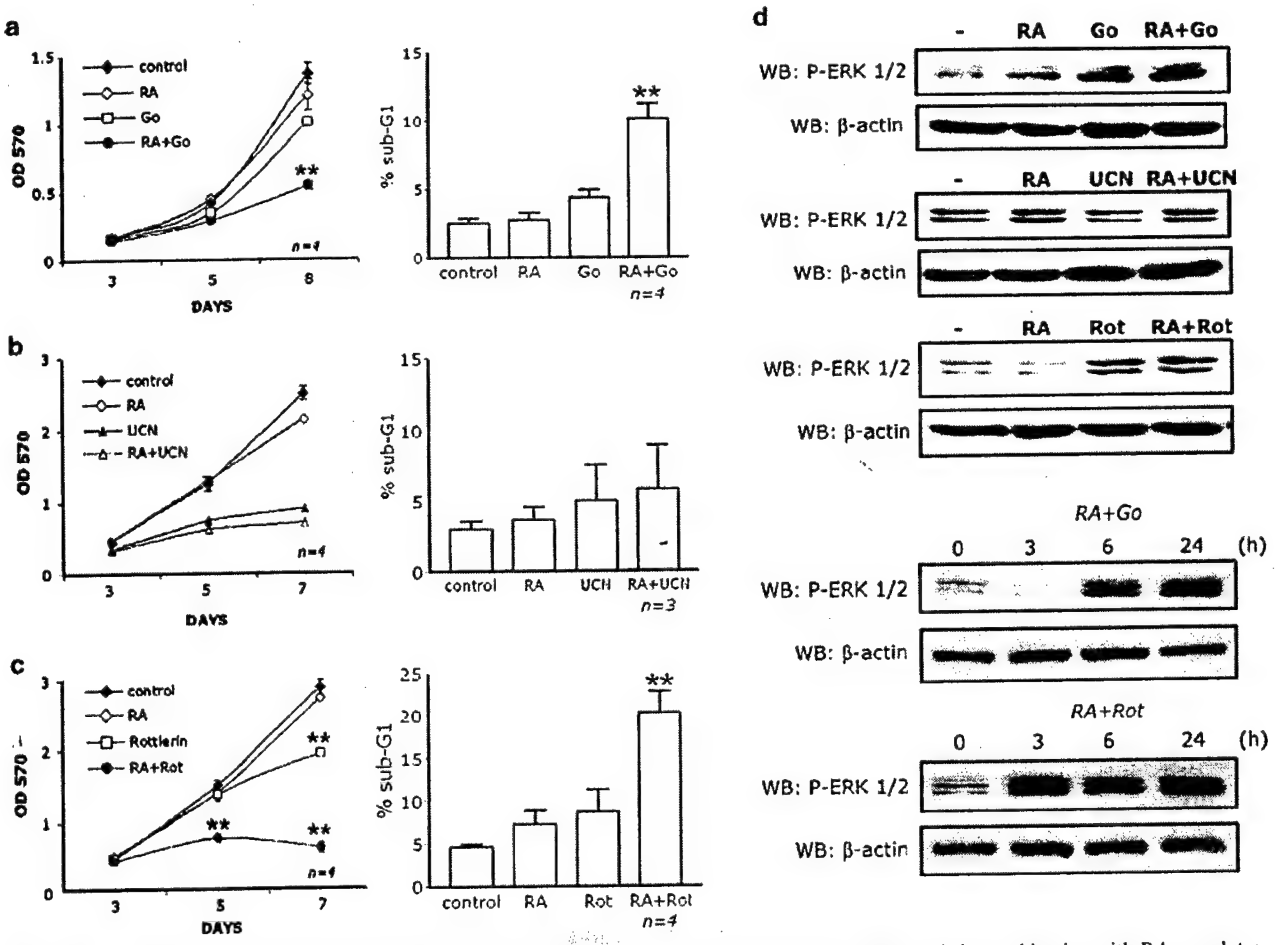

Figure 8 Ability of three PKC inhibitors to inhibit MDA-MB-231 cell proliferation and induce apoptosis in combination with RA correlates with their ability to activate ERK. Cell number and apoptosis were assessed by SRB staining and PI staining, respectively. (**a**) Cells were treated with 10 μM RA and 1 μM Go6983 (**b**) Cells were treated with 10 μM RA and 10 nM UCN-01. (**c**) Cells were treated with 10 μM RA and 0.5 μM Rottlerin. (**d**) Activation of ERK was detected by Western blotting in whole cell lysates from cells treated for 72 h with each of the inhibitors in the absence or presence of RA (panels 1, 3 and 5) as well as cells treated with RA + Go or RA + Rot for 3–24 h (panels 7 and 9). For each of the blots, β-actin was analysed as a loading control. **Indicates a significant difference compared to *all* treatments ($P<0.01$)

literature concerning expression of the different PKC isoforms in MDA-MB-231. While some authors see no expression of PKCα (Cho *et al*., 1997), most reports show that PKCα is the main isoform expressed in these cells (Platet *et al*., 1998; Gauthier *et al*., 2003). In agreement with these reports, our MDA-MB-231 express high levels of PKCα, as well as lower levels of PKCδ. We showed that GF109203X decreases the total levels of both PKCα and δ, and that RA further potentiates this decrease (Figure 4). Treatments (48–72 h) also cause a significant decrease in levels of phospho-PKC (Figures 4 and 6). While induction of both PKCα and δ expression and activity by RA has been shown in other cell lines, including RA-sensitive breast cancer cells (Cho *et al*., 1997; Cho and Talmage, 2001; Kambhampati *et al*., 2003), we observed a small decrease in PKCα and no effect on PKCδ after treatment with RA alone.

Inhibitors of PKC have previously been shown to sensitize cells to apoptosis induced by different cytotoxic drugs (Shao *et al*., 1997; Huigsloot *et al*., 2003) and many PKC inhibitors that are currently under development for use in cancer treatment are themselves potent inducers of apoptosis (Shao *et al*., 1997; Gschwend *et al*., 2000; Tenzer *et al*., 2001). GF is not a strong inducer of apoptosis, but has been shown to potentiate the cytotoxic effects of tumor necrosis factor (TNF) (Basu *et al*., 2001) and Heregulin (Le *et al*., 2001) in breast cancer cells, as well as anti-CD95-induced apoptosis in Jurkat T cells (Drew *et al*., 1998). In the case of TNF, enhancement of apoptosis by GF occurred in several cell lines, including MDA-MB-231, and was linked to cleavage of PKCδ and ε (Basu *et al*., 2001). Conversely, potentiation of Heregulin-induced apoptosis in the SKBr3 cell line was proposed to be associated with impairment of PKCα activity (Le *et al*., 2001). In agreement with this latter report, we observed decreased

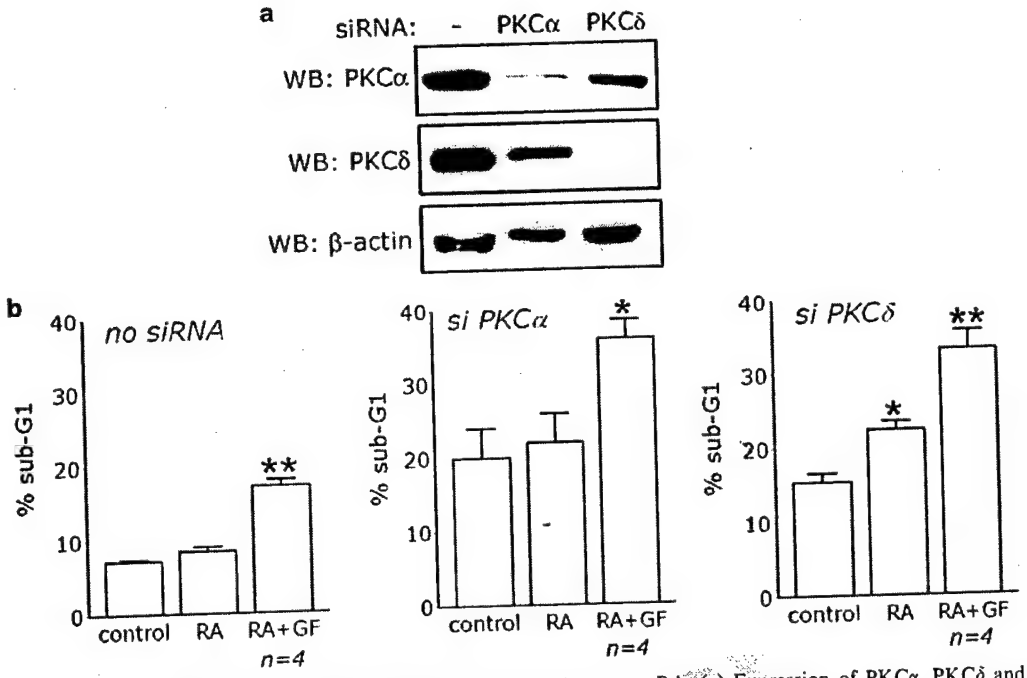

Figure 9 Downregulation of PKCδ using siRNA confers partial responsiveness to RA. (**a**) Expression of PKCα, PKCδ and β-actin was assessed by Western blotting 72 h post-siRNA transfection and shows isoform-selective downregulation. (**b**) Cells transfected with siRNA were treated with 10 μM RA ± 1 μM GF for 72 h and apoptosis was assessed by PI staining. 'No siRNA' indicates cells subjected to the transfection procedure without RNA. $*P<0.05$ and $**P<0.01$ indicate significant differences compared to respective controls

expression of both the α and δ isoform after treatment with GF. Thus, inhibition of PKCα and/or δ may potentially be important for the ability of GF to induce cell death in combination with RA and other retinoids.

Three of the PKC inhibitors used in this study, GF, Go and UCN-01, are broad-range inhibitors that affect most PKC isoforms. However, there are some differences between these inhibitors, both structural and functional. UCN-01 is more selective for the cPKCs (α, β, γ) than GF and Go (Hofmann, 2001), and also inhibits cyclin-dependent kinases, PDK-1 and CHK-1 (Kawakami *et al*., 1996; Busby *et al*., 2000; Graves *et al*., 2000; Sato *et al*., 2002; Sausville, 2003). In fact, inhibition of cyclin-dependent kinases may explain why UCN-01 was found to have a strong antiproliferative effect in MDA-MB-231 cells, but did not induce apoptosis (see Figure 8b).

Under our experimental conditions, GF, Go and UCN-01 all decreased the level of phosphorylated PKCα, the main isoform expressed in MDA-MB-231, but only GF and Go caused growth inhibition and apoptosis in combination with RA. Thus, it is clear that decreasing PKCα alone is not sufficient to promote retinoid-induced apoptosis. We went on to test if the PKCδ selective inhibitor Rottlerin could interact with RA to induce growth inhibition and apoptosis. We found that it did, which may indicate a role for PKCδ.

The role of PKCδ in apoptosis is complicated, and there are numerous reports of it acting both as a proapoptotic and an antiapoptotic factor, presumably depending on the apoptotic stimuli as well as the cell type (Li *et al*., 1999; Mandil *et al*., 2001; Brodie and Blumberg, 2003; Clark *et al*., 2003; Ni *et al*., 2003). Importantly, PKCδ was recently reported to be a prosurvival factor in human breast cancer cell lines (McCracken *et al*., 2003). The authors of that study showed that Rottlerin, as well as expression of a dominant-negative PKCδ or a PKCδ antisense, reduced cell survival in both MDA-MB-231 and MCF-7 cells. The PKCδ antisense also sensitized the cells to the apoptotic effects of gamma-radiation. Thus, it is possible that the apoptosis observed in MDA-MB-231 cells upon treatment with RA + GF, RA + Go or RA + Rottlerin is caused, at least partially, by reduced PKCδ activity or levels. This conclusion was supported by siRNA studies, where a PKCδ siRNA selectively conferred enhanced sensitivity to RA (see Figure 9). However, Rottlerin has also been reported to cause cell death independently of PKCδ (Tillman *et al*., 2003), and it cannot be ruled out that GF and Go may also stimulate apoptotic pathways independently of their effects on PKC. Again, our siRNA studies supported this possibility. Although close to complete knockout of PKCδ was achieved, and this caused an increase in RA response, addition of GF still significantly enhanced the effect. This strongly suggests that GF (as well as Go and Rottlerin) has an effect in addition to inhibition of PKC, which is required for full stimulation of retinoid-induced apoptosis. Our evidence further suggests that this effect entails activation of ERK, since GF, Go and Rottlerin (but not UCN-01) all induce an early and sustained activation of ERK.

In fact, we found that levels of both phospho-ERK and phospho-p38 MAPK are significantly increased by GF. Activation of ERK occurs after as little as 3 h of treatment and is sustained for at least 72 h, whereas p38 is significantly activated only after 24 h. Moreover, inhibition of ERK, but not p38, almost completely suppresses induction of apoptosis by RA + GF. This was unexpected, since ERK activation is usually associated with cell proliferation and not cell death (Johnson and Lapadat, 2002; Santen *et al.*, 2002). However, there are some reports in the literature of ERK acting as a proapoptotic molecule. For example, DNA-damaging agents such as etoposide, doxorubicin, cisplatin and UV radiation cause activation of ERK. This activation leads to either induction of the cyclin-dependent kinase inhibitor p21 and cell growth arrest, or apoptosis, and both can be inhibited by the MEK inhibitors PD98059 and U0126 (Persons *et al.*, 2000; Wang *et al.*, 2000; Tang *et al.*, 2002; Lee *et al.*, 2003). Likewise, Resveratrol, a phytoalexin found in red wine, has been reported to induce apoptosis in various cancer cell types in an ERK-dependent manner (She *et al.*, 2001; Lin *et al.*, 2002; Shih *et al.*, 2002).

Crosstalk between the ERK pathway and retinoid signaling has been described previously, although the details of this crosstalk are far from clear and appear to be cell type dependent. There are several reports showing that ERK can inhibit retinoid signaling through phosphorylation of RXRα, which interferes with its transactivation function (Solomon *et al.*, 1999; Matsushima-Nishiwaki *et al.*, 2001; Adachi *et al.*, 2002). Retinoids also inhibit ERK activity in several cell lines, and this has been associated with growth inhibition (Sah *et al.*, 2002; Crowe *et al.*, 2003). On the other hand, activation of ERK by RA is required for its ability to induce differentiation of HL-60 cells (Yen *et al.*, 1998; Miranda *et al.*, 2002) as well as differentiation of embryonic stem cells into adipocytes (Bost *et al.*, 2002).

To our knowledge, this report is the first example of ERK stimulating induction of apoptosis by RA. Although RA is known to cause growth arrest and differentiation in many tumor cell lines (Jiang *et al.*, 1994; Giannini *et al.*, 1997; Rosenauer *et al.*, 1998; Hsu *et al.*, 2000), there are numerous cell types, including breast cancer cells, where RA and other retinoids also induce apoptosis (Nagy *et al.*, 1995; Liu *et al.*, 1996; Spanjaard *et al.*, 1997; Mangiarotti *et al.*, 1998; Lee *et al.*, 2000b; Pettersson *et al.*, 2002). While activation of ERK has been implicated in apoptosis induced by other stimuli (Persons *et al.*, 2000; Wang *et al.*, 2000; Tang *et al.*, 2002; Lee *et al.*, 2003), as well as induction of differentiation by RA (Yen *et al.*, 1998; Miranda *et al.*, 2002), we show that RA in combination with several PKC inhibitors induces apoptosis in RA-resistant cells, through a mechanism that involves activation of ERK. The lack of response to UCN-01 + RA can be explained by the fact that UCN-01 neither inhibits PKCδ nor activates ERK in these cells.

It is unclear how inhibitors of PKC can activate MAPK signaling, but the fact that ERK is activated early, before any effects on PKC were observed (Figure 6), may suggest that a PKC-independent mechanism is responsible. Our studies using siRNA also showed that simply downregulating PKCα or δ did not affect ERK activity (data not shown). Thus, further studies will be necessary in order to elucidate the mechanism. It is also not clear how RA and GF regulate PKC levels. At the RNA level, we observed only a weak downregulation of PKCα and no effect on PKCδ. This indicates that the main regulation occurs at the protein level, suggesting that either the rate of protein synthesis is reduced or protein degradation is stimulated. PKCα and δ are both subject to degradation via the proteasome, and PKCδ is also a target of caspases and is cleaved during apoptosis (Lee *et al.*, 1996; Denning *et al.*, 1998; Lu *et al.*, 1998). Therefore, it is possible that the observed decrease in PKCα and δ, as well as phospho-PKC, is a result of the fact that the cells are undergoing apoptosis after treatment with RA + GF. In support of this, the decrease is only seen after 48–72 h, a time when the cells are starting to die. Interestingly, the MEK inhibitors PD and U0 were able to rescue the decrease in both total and phosphorylated PKC (Figure 7 and data not shown). This may of course imply that activation of MEK/ERK is necessary for degradation of PKC, but is perhaps more likely to reflect the fact that PD and U0 block apoptosis. Thus, in summary, the ability of the PKC inhibitors to stimulate retinoid-induced apoptosis may require inhibition/downregulation of PKC, and appear to involve PKCδ in particular. In addition, a sustained activation of ERK1/2 is required, but further studies will be needed to elucidate the link between these events.

Although retinoids have shown promising preclinical antitumor activity in numerous models, successful clinical use has been mainly limited to APL, a disease characterized by expression of fusion oncoproteins invariably involving RARα (Mann *et al.*, 2001). Numerous synthetic retinoids with a more favorable activity than RA have been developed, some of which are in clinical trials from which the outcome is yet to be reported. In order to increase the activity of retinoids against breast cancer, and particularly ER-negative disease, we examined the possibility of combination therapy using kinase inhibitors. Our results showing a strong growth inhibitory response to RA in combination with several PKC inhibitors are encouraging, especially since two synthetic retinoids, TTNPB and Bexarotene, also cause apoptosis in MDA-MB-231 when combined with GF. In view of recent advances that have been made in the development of cancer therapies that specifically target various signal transduction pathways, further research is warranted to investigate whether the combination of retinoids with such drugs may enhance the activity of retinoids in the clinical treatment of breast cancer.

Materials and methods

Drugs and reagents

The PKC inhibitors GF109203X, Go6983 and Rottlerin, the p38MAPK inhibitor SB203580 and the MEK inhibitor U0126 were purchased from Calbiochem. The MEK inhibitor PD98059 was purchased from Sigma. The JNK inhibitor SP600125 was purchased from Biomol. UCN-01 was obtained from Dr RJ Schultz at the Drug synthesis and Chemistry Branch, NCI, Bethesda, MD, USA. The compounds were dissolved in DMSO and kept at −20°C. All-*trans*-retinoic acid was purchased from Sigma and TTNPB (LG100272) and Bexarotene (Targretin®, LGD1069) were obtained from Ligand Pharmaceuticals Inc., San Diego, CA, USA. The retinoids were dissolved in DMSO at a concentration of 10 mM and stored in the dark at −70°C.

Cell culture

MDA-MB-231 cells (ATCC) were routinely maintained in alpha-MEM (Invitrogen) supplemented with 5% fetal bovine serum (FBS) at 37°C in a humidified atmosphere containing 5% CO_2. During all treatment experiments, cells were cultured in phenol red-free alpha-MEM with 2 or 5% charcoal-stripped serum (CSS).

Cell proliferation studies

Cells were seeded in 24-well plates at a density of 2000 cells/well. The next day, fresh media containing DMSO or the inhibitory compounds was added. On the days indicated, cells were fixed in 10% trichloroacetic acid and subsequently stained with sulforhodamine B (SRB, Sigma). SRB is an aminoxanthene dye, which binds to basic amino-acid residues and gives an index of culture cell protein that is linear with cell number (Skehan *et al*., 1990). Bound SRB was solubilized in 10 mM unbuffered Tris and optical density was measured at 570 nm in a microplate reader.

Cell cycle analysis and apoptosis assays

For these experiments, cells were treated with 10 μM RA and 1 μM GF109203X for 72 h, unless otherwise indicated. Propidium iodide (PI) staining followed by flow cytometry was used to analyse distribution of the cells in the different phases of the cell cycle, as well as to detect cells with a sub-G1 DNA content. Cells were seeded in six-well plates at a density of 5×10^4 cells/well and the next day fresh media containing DMSO or the inhibitory compounds was added. On day 3, cells were trypsinized, washed twice with sample buffer (PBS + 1g/l glucose) and fixed in 70% ethanol at a density of 5×10^5–1×10^6 cells/ml. After a minimum of 18 h, cells were washed with sample buffer, resuspended in PI staining solution containing 50 μg/ml PI and incubated at room temperature for 30 min. Fluorescence was measured on a Becton-Dickinson FACSCalibur. Cell cycle analysis was performed using ModFit LT software and cells with sub-G1 DNA content were quantified using CellQUEST software.

Activation of caspase-3 was detected using a fluorescent caspase-3 inhibitor, Red-DEVD-FMK (Oncogene), which irreversibly binds to activated caspase-3 in apoptotic cells. Treated cells were trypsinized, resuspended in 300 μl culture media and incubated in the presence of 1 μl Red-DEVD-FMK for 1 h at 37°C. Cells were then washed, resuspended and analysed by flow cytometry, using the FL-2 channel.

Apoptosis was also assessed using Annexin V-FITC staining (BD Biosciences), according to the protocol recommended by the manufacturer. Briefly, cells treated as indicated were trypsinized, washed twice with PBS, resuspended in 1 × binding buffer and stained with Annexin V-FITC and PI for 15 min in the dark. Fluorescence was measured on a Becton-Dickinson FACSCalibur and FITC *vs* PI dotblots were analysed using CellQuest software.

Transient transfections

Cells were seeded into six-well plates at a density of 5×10^4 cells/well. The next day, fresh media containing 5% CSS was added and cells were pretreated with GF (1 μM) or vehicle (DMSO). After 48 h, fresh media containing no treatment was added and the cells were cotransfected with βRARE-tk-CAT or CRBPII-tk-CAT (1 μg/well) plus pCMV-βgal (1 μg/well), using FuGENE transfection reagent (Roche Biochemicals) as specified by the manufacturer. At 4–5 h post-transfection, RA (1 μM), GF (1 μM), RA + GF, or vehicle was added and the cells were harvested 48 h post-transfection. β-Gal activity and CAT activity were assayed as previously described (Rosenauer *et al*., 1998).

Western blotting

Whole cell lysates were prepared by washing the cells in ice-cold PBS, resuspending them in lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Triton X-100 plus protease and phosphatase inhibitors) followed by incubation on ice and microcentrifugation at maximum speed for 10 min to exclude insoluble material. Protein concentrations were determined by the Bradford assay (BioRad). Equal amounts of protein (15–80 μg) were electrophoretically separated in 10% SDS–polyacrylamide gels and proteins were immobilized by transfer onto nitrocellulose membranes. Membranes were immunoprobed with antibodies to PKCα (Transduction laboratories), PKCδ (Santa Cruz), phospho-PKC, phospho-ERK and phospho-p38MAPK (Cell Signaling Technologies), ERK2 (SantaCruz), PARP (Oncogene) and β-actin (Sigma) followed by a secondary, peroxidase-labeled antibody. The proteins were visualized by enhanced chemiluminescence (ECL, Amersham Biosciences).

Kinase assay

Whole cell extracts were prepared as described above. For assessment of JNK activity, 200 μg of protein extracts were subjected to immunoprecipitation with an antibody specific to JNK1 (Santa Cruz). The cell extracts were incubated with 1 μg of the antibody and 20 μl packed Protein A sepharose CL-4B beads (Amersham) in a total volume of 200 μl for 1 h at 4°C. The immunoprecipitates were washed with 1 × kinase buffer (25 mM HEPES pH 7.5, 25 mM β-glycerophosphate, 25 mM $MgCl_2$, 1 mM $NaOV_4$, 1 mM DTT) and subsequently incubated with 1 μg GST-c-Jun in 2 × kinase buffer containing 2 μCi [^{32}P]γ-ATP. The reaction was terminated by addition of binding buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 0.1% NP-40) followed by centrifugation at maximum speed for 1 min. The supernatant was transferred to a new tube and GST-c-Jun was pulled down by incubation with Glutathione sepharose-4B beads (Amersham) for 1 h at 4°C. The beads were washed three times with binding buffer, resuspended in SDS–PAGE loading buffer and boiled for 5 min. Supernatants were separated by SDS–PAGE and phosphorylated c-Jun was visualized by autoradiography.

RNA interference

21-nucleotide siRNAs (HPP grade) were chemically synthesized by Xeragon oligonucleotides (Xeragon–Qiagen). The sequences of each siRNA pairs were as follows: PKCα 5′-AAA GGC UGA GGU UGC UGA UTT-3′ and 5′-AUC AGC AAC CUC AGC CUU UTT-3′; PKCδ 5′-CGA CAA GAU CAU CGG CAG ATT-3′ and 5′-UCU GCC GAU GAU CUU GUC GTT-3′ (Irie *et al*., 2002). A measure of 20 μM siRNA duplexes were generated by annealing of sense and antisense nucleotides as specified by the supplier and transfection was performed using TransMessenger™ transfection reagent (Qiagen). Cells were seeded in six-well plates at a density of 1×10^5 cells/well. The next day, cells were transfected with siRNA (4 μg/well) as recommended by the manufacturer, using a ratio of RNA:TransMessenger™ of 1:3. After a 2.5 h incubation period, transfection complexes were removed, the cells washed twice with PBS, and regular growth media added. For apoptosis assessment, drugs were added after another 3–4 h and cells were harvested for PI staining and flow cytometry analysis 72 h post-transfection.

Statistics

All experiments were performed at least three times and results from representative experiments are shown as means of the number of replicates indicated in each figure. To evaluate statistical significance for growth, apoptosis and transcription experiments, one-way ANOVA with Dunnett's post-test was performed using GraphPad Prism version 3.0. Significant differences compared to control (untreated cells) are shown, unless otherwise stated. $*P<0.05$, $**P<0.01$.

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References

Adachi S, Okuno M, Matsushima-Nishiwaki R, Takano Y, Kojima S, Friedman SL, Moriwaki H and Okano Y. (2002). *Hepatology*, **35**, 332–340.

Adan Y, Goldman Y, Haimovitz R, Mammon K, Eilon T, Tal S, Tene A, Karmel Y and Shinitzky M. (2003). *Cancer Lett.*, **194**, 67–79.

Appierto V, Villani MG, Cavadini E, Lotan R, Vinson C and Formelli F. (2004). *Cell Death Differ.*, **11**, 270–279.

Basu A, Mohanty S and Sun B. (2001). *Biochem. Biophys. Res. Commun.*, **280**, 883–891.

Boskovic G, Desai D and Niles RM. (2002). *J. Biol. Chem.*, **277**, 2611–2619.

Bost F, Caron L, Marchetti I, Dani C, Le Marchand-Brustel Y and Binetruy B. (2002). *Biochem. J.*, **361**, 621–627.

Brodie C and Blumberg PM. (2003). *Apoptosis*, **8**, 19–27.

Busby EC, Leistritz DF, Abraham RT, Karnitz LM and Sarkaria JN. (2000). *Cancer Res.*, **60**, 2108–2112.

Cho Y and Talmage DA. (2001). *Exp. Cell Res.*, **269**, 97–108.

Cho Y, Tighe AP and Talmage DA. (1997). *J. Cell Physiol.*, **172**, 306–313.

Clark AS, West KA, Blumberg PM and Dennis PA. (2003). *Cancer Res.*, **63**, 780–786.

Crowe DL, Kim R and Chandraratna RA. (2003). *Mol. Cancer Res.*, **1**, 532–540.

Delia D, Aiello A, Formelli F, Fontanella E, Costa A, Miyashita T, Reed JC and Pierotti MA. (1995). *Blood*, **85**, 359–367.

Delmotte M-H, Tahayato A, Formstecher P and Lefebvre P. (1999). *J. Biol. Chem.*, **274**, 38225–38231.

Denning MF, Wang Y, Nickoloff BJ and Wrone-Smith T. (1998). *J. Biol. Chem.*, **273**, 29995–30002.

del Rincon SV, Rousseau C, Samanta R and Miller Jr WH. (2003). *Oncogene*, **22**, 3353–3360.

De Luca LM, Darwiche N, Jones CS and Scita G. (1995). *Sci. Am. – Sci. Med.*, **2**, 28–37.

Drew L, Kumar R, Bandyopadhyay D and Gupta S. (1998). *Int. Immunol.*, **10**, 877–889.

Evans RM. (1988). *Science*, **240**, 889–895.

Fitzgerald P, Teng M, Chandraratna RAS, Heyman RA and Allegretto EA. (1997). *Cancer Res.*, **57**, 2642–2650.

Gauthier ML, Torretto C, Ly J, Francescutti V and O'Day DH. (2003). *Biochem. Biophys. Res. Commun.*, **307**, 839–846.

Giannini G, Dawson MI, Zhang X and Thiele CJ. (1997). *J. Biol. Chem.*, **272**, 26693–26701.

Graves PR, Yu L, Schwarz JK, Gales J, Sausville EA, O'Connor PM and Piwnica-Worms H. (2000). *J. Biol. Chem.*, **275**, 5600–5605.

Gschwendt M, Dieterich S, Rennecke J, Kittstein W, Mueller HJ and Johannes FJ. (1996). *FEBS Lett.*, **392**, 77–80.

Gschwend JE, Fair WR and Powell CT. (2000). *Mol. Pharmacol.*, **57**, 1224–1234.

Gschwendt M, Muller HJ, Kielbassa K, Zang R, Kittstein W, Rincke G and Marks F. (1994). *Biochem. Biophys. Res. Commun.*, **199**, 93–98.

Hail Jr N, Youssef EM and Lotan R. (2001). *Cancer Res.*, **61**, 6698–6702.

Hofmann J. (2001). *Rev. Physiol. Biochem. Pharmacol.*, **142**, 1–96.

Hsu SL, Hsu JW, Liu MC, Chen LY and Chang CD. (2000). *Exp. Cell Res.*, **258**, 322–331.

Huigsloot M, Tijdens RB and van de Water B. (2003). *Mol. Pharmacol.*, **64**, 965–973.

Irie N, Sakai N, Ueyama T, Kajimoto T, Shirai Y and Saito N. (2002). *Biochem. Biophys. Res. Commun.*, **298**, 738–743.

Jiang H, Lin J, Su ZZ, Collart FR, Huberman E and Fisher PB. (1994). *Oncogene*, **9**, 3397–3406.

Johnson GL and Lapadat R. (2002). *Science*, **298**, 1911–1912.

Kambhampati S, Li Y, Verma A, Sassano A, Majchrzak B, Deb DK, Parmar S, Giafis N, Kalvakolanu DV, Rahman A, Uddin S, Minucci S, Tallman MS, Fish EN and Platanias LC. (2003). *J. Biol. Chem.*, **278**, 32544–32551.

Kawakami K, Futami H, Takahara J and Yamaguchi K. (1996). *Biochem. Biophys. Res. Commun.*, **219**, 778–783.

Keranen LM, Dutil EM and Newton AC. (1995). *Curr. Biol.*, **5**, 1394–1403.

Kitareewan S, Spinella MJ, Allopenna J, Reczek PR and Dmitrovsky E. (1999). *Oncogene*, **18**, 5747–5755.

Kizaki M, Dawson MI, Heyman R, Elster E, Morosetti R, Pakkala S, Chen DL, Ueno H, Chao W, Morikawa M, Ikeda Y, Heber D, Pfahl M and Koeffler HP. (1996). *Blood*, **87**, 1977–1984.

Lee H-J, Suh Y-A, Robinson MJ, Clifford JL, Hong WK, Woodgett JR, Cobbs MH, Mangelsdorf DJ and Kurie JM. (2000a). *J. Biol. Chem.*, **275**, 32193–32199.

Lee HW, Smith L, Pettit GR, Vinitsky A and Smith JB. (1996). *J. Biol. Chem.*, **271,** 20973–20976.
Lee MO, Han SY, Jiang S, Park JH and Kim SJ. (2000b). *Biochem. Pharmacol.*, **59,** 485–496.
Lee YJ, Soh JW, Jeoung DI, Cho CK, Jhon GJ, Lee SJ and Lee YS. (2003). *Biochim. Biophys. Acta*, **1593,** 219–229.
Le XF, Marcelli M, McWatters A, Nan B, Mills GB, O'Brian CA and Bast Jr RC. (2001). *Oncogene*, **20,** 8258–8269.
Le XF, McWatters A, Wiener J, Wu JY, Mills GB and Bast Jr RC. (2000). *Clin. Cancer Res.*, **6,** 260–270.
Li D, Zimmerman TL, Thevananther S, Lee HY, Kurie JM and Karpen SJ. (2002). *J. Biol. Chem.*, **277,** 31416–31422.
Li L, Lorenzo PS, Bogi K, Blumberg PM and Yuspa SH. (1999). *Mol. Cell. Biol.*, **19,** 8547–8558.
Lin HY, Shih A, Davis FB, Tang HY, Martino LJ, Bennett JA and Davis PJ. (2002). *J. Urol.*, **168,** 748–755.
Lippman SC, Heyman RA, Kurie JM, Benner SE and Hong WK. (1995). *J. Cell Biochem.*, (Suppl 22), 1–10.
Liu Y LM, Wang HG, Li Y, Hashimoto Y, Klaus M, Reed JC and Zhang X. (1996). *Mol. Cell. Biol.*, **16,** 1138–1149.
Lotan R. (1996). *FASEB J.*, **10,** 1031–1039.
Lu Z, Liu D, Hornia A, Devonish W, Pagano M and Foster DA. (1998). *Mol. Cell. Biol.*, **18,** 839–845.
Mandil R, Ashkenazi E, Blass M, Kronfeld I, Kazimirsky G, Rosenthal G, Umansky F, Lorenzo PS, Blumberg PM and Brodie C. (2001). *Cancer Res.*, **61,** 4612–4619.
Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schuts G, Blumberg B, Kastner P, Mark M, Chambon P and Evans R. (1995). *Cell*, **83,** 835–839.
Mangelsdorf DJ, Umesono K, Kliewer SA, Borgmeyer U, Ong ES and Evans RM. (1991). *Cell*, **66,** 555–561.
Mangiarotti R, Danova M and Alberici C. (1998). *Br. J. Cancer*, **77,** 186–191.
Mann KK, Shao W and Miller Jr WH. (2001). *Curr. Oncol. Rep.*, **3,** 209–216.
Marchetti P, Zamzami N, Joseph B, Schraen-Maschke S, Méreau-Richard C, Costantini P, Métivier D, Susin SA, Kroemer G and Formstecher P. (1999). *Cancer Res.*, **59,** 6257–6266.
Martelli AM, Sang N, Borgatti P, Capitani S and Neri LM. (1999). *J. Cell Biochem.*, **74,** 499–521.
Matsushima-Nishiwaki R, Okuno M, Adachi S, Sano T, Akita K, Moriwaki H, Friedman SL and Kojima S. (2001). *Cancer Res.*, **61,** 7675–7682.
McCracken MA, Miraglia LJ, McKay RA and Strobl JS. (2003). *Mol. Cancer Ther.*, **2,** 273–281.
Mellor H and Parker PJ. (1998). *Biochem. J.*, **332** (Part 2), 281–292.
Miller WH. (1998). *Cancer*, **83,** 1471–1482.
Miranda MB, McGuire TF and Johnson DE. (2002). *Leukemia*, **16,** 683–692.
Nagy L, Thomazy VA, Shipley GL, Fesus L, Lamph W, Heyman RA, Chandraratna RA and Davies PJ. (1995). *Mol. Cell. Biol.*, **15,** 3540–3551.
Ni H, Ergin M, Tibudan SS, Denning MF, Izban KF and Alkan S. (2003). *Br. J. Haematol.*, **121,** 849–856.
Ortiz MA, Lopez-Hernandez FJ, Bayon Y, Pfahl M and Piedrafita FJ. (2001). *Cancer Res.*, **61,** 8504–8512.
Parekh DB, Ziegler W and Parker PJ. (2000). *EMBO J.*, **19,** 496–503.
Pepper C, Ali K, Thomas A, Hoy T, Fegan C, Chowdary P, Kell J and Bentley P. (2002). *Eur. J. Haematol.*, **69,** 227–235.
Persons DL, Yazlovitskaya EM and Pelling JC. (2000). *J. Biol. Chem.*, **275,** 35778–35785.
Pettersson F, Dalgleish AG, Bissonnette RP and Colston KW. (2002). *Br. J. Cancer*, **87,** 555–561.
Platet N, Prevostel C, Derocq D, Joubert D, Rochefort H and Garcia M. (1998). *Int. J. Cancer*, **75,** 750–756.
Radominska-Pandya A, Chen G, Czernik PJ, Little JM, Samokyszyn VM, Carter CA and Nowak G. (2000). *J. Biol. Chem.*, **275,** 22324–22330.
Rigobello MP, Scutari G, Friso A, Barzon E, Artusi S and Bindoli A. (1999). *Biochem. Pharmacol.*, **58,** 665–670.
Rochefort H, Glondu M, Sahla ME, Platet N and Garcia M. (2003). *Endocr. Relat. Cancer*, **10,** 261–266.
Rosenauer A, Nervi C, Davison K, Lamph WW, Mader S and Miller Jr WH. (1998). *Cancer Res.*, **58,** 5110–5116.
Rousseau C, Pettersson F, Couture MC, Paquin A, Galipeau J, Mader S and Miller WH. (2003). *J. Steroid Biochem. Mol. Biol.*, **86,** 1–14.
Rubin M, Fenig E, Rosenauer A, Menendez-Botet C, Achkar C, Bentel JM, Yahalom J, Mendelsohn J and Miller Jr WH. (1994). *Cancer Res.*, **54,** 6549–6556.
Sah JF, Eckert RL, Chandraratna RA and Rorke EA. (2002). *J. Biol. Chem.*, **277,** 9728–9735.
Santen RJ, Song RX, McPherson R, Kumar R, Adam L, Jeng MH and Yue W. (2002). *J. Steroid Biochem. Mol. Biol.*, **80,** 239–256.
Sato S, Fujita N and Tsuruo T. (2002). *Oncogene*, **21,** 1727–1738.
Sausville EA. (2003). *Curr. Med. Chem. Anti-Cancer Agents*, **3,** 47–56.
Shao RG, Shimizu T and Pommier Y. (1997). *Exp. Cell Res.*, **234,** 388–397.
Sheikh MS, Shao ZM, Chen JC, Hussain A, Jetten AM and Fontana JA. (1993). *J. Cell Biochem.*, **53,** 394–404.
Sheikh MS, Shao ZM, Li XS, Dawson M, Jetten AM, Wu S, Conley BA, Garcia M, Rochefort H and Fontana JA. (1994). *J. Biol. Chem.*, **269,** 21440–21447.
She QB, Bode AM, Ma WY, Chen NY and Dong Z. (2001). *Cancer Res.*, **61,** 1604–1610.
Shih A, Davis FB, Lin HY and Davis PJ. (2002). *J. Clin. Endocrinol. Metab.*, **87,** 1223–1232.
Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, Warren JT, Bokesh H, Kenney S and Boyd MR. (1990). *J. Natl. Cancer Inst.*, **82,** 1107–1112.
Solomon C, White JH and Kremer R. (1999). *J. Clin. Invest.*, **103,** 1729–1735.
Spanjaard RA, Ikeda M, Lee PJ, Charpentier B, Chin WW and Eberlein TJ. (1997). *J. Biol. Chem.*, **272,** 18990–18999.
Sun SY, Li W, Yue P, Lippman SM, Hong WK and Lotan R. (1999a). *Cancer Res.*, **59,** 2493–2498.
Sun SY, Yue P, Wu GS, El-Deiry WS, Shroot B, Hong WK and Lotan R. (1999b). *Oncogene*, **18,** 2357–2365.
Tang D, Wu D, Hirao A, Lahti JM, Liu L, Mazza B, Kidd VJ, Mak TW and Ingram AJ. (2002). *J. Biol. Chem.*, **277,** 12710–12717.
Tenzer A, Zingg D, Rocha S, Hemmings B, Fabbro D, Glanzmann C, Schubiger PA, Bodis S and Pruschy M. (2001). *Cancer Res.*, **61,** 8203–8210.
Tillman DM, Izeradjene K, Szucs KS, Douglas L and Houghton JA. (2003). *Cancer Res.*, **63,** 5118–5125.
Toullec D, Pianetti P, Coste H, Bellevergue P, Grand-Perret T, Ajakane M, Baudet V, Boissin P, Boursier E and Loriolle F. (1991). *J. Biol. Chem.*, **266,** 15771–15781.
van der Burg B, Slager-Davidov R, van der Leede BM, de Laat SW and van der Saag PT. (1995). *Mol. Cell. Endocrinol.*, **112,** 143–152.
van der Burg B, van der Leede BM, Kwakkenbos-Isbrucker L, Salverda S, de Laat SW and van der Saag PT. (1993). *Mol. Cell. Endocrinol.*, **91,** 149–157.

Wang X, Martindale JL and Holbrook NJ. (2000). *J. Biol. Chem.*, **275**, 39435–39443.

Wu JM, DiPietrantonio AM and Hsieh TC. (2001). *Apoptosis*, **6**, 377–388.

Yen A, Roberson MS, Varvayanis S and Lee AT. (1998). *Cancer Res.*, **58**, 3163–3172.

Yung BY and Hui EK. (1995). *J. Biomed. Sci.*, **2**, 154–159.

Zhao X, Demary K, Wong L, Vaziri C, McKenzie AB, Eberlein TJ and Spanjaard RA. (2001). *Cell Death Differ.*, **8**, 878–886.

Zhu WY, Jones CS, Kiss A, Matsukuma K, Amin S and De Luca LM. (1997). *Exp. Cell Res.*, **234**, 293–299.